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MINIREVIEW

Accessing the Inaccessible: Molecular Tools for Bifidobacteria

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Bifidobacteria are an important group of the human intestinal microbiota that have been shown to exert a number of beneficial probiotic effects on the health status of their host. Due to these effects, bifidobacteria have attracted strong interest in health care and food industries for probiotic applications and several species are listed as so-called "generally recognized as safe" (GRAS) microorganisms. Moreover, recent studies have pointed out their potential as an alternative or supplementary strategy in tumor therapy or as live vaccines. In order to study the mechanisms by which these organisms exert their beneficial effects and to generate recombinant strains that can be used as drug delivery vectors or live vaccines, appropriate molecular tools are indispensable. This review provides an overview of the currently available methods and tools to generate recombinant strains of bifidobacteria. The currently used protocols for transformation of bifidobacteria, as well as replicons, selection markers, and determinants of expression, will be summarized. We will further discuss promoters, terminators, and localization signals that have been used for successful generation of expression vectors.

At present, 39 species with a total of 7 subspecies are assigned to the genus *Bifidobacterium* (63). In the publically accessible microbial genome databases of the National Center for Biotechnology Information, European Bioinformatics Institute, and Genomes OnLine Database 23, completely sequenced and annotated genomes are available. Moreover, the more comprehensive Genomes OnLine Database lists 14 bifidobacterial genomes as permanent drafts, 47 incomplete, and 12 targeted sequencing projects. Complete sequenced and annotated genomes are available for strains of the following species: *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. dentium*, and *B. longum.* Except for *B. dentium*, which is frequently isolated from the oral cavity, bifidobacteria are commonly observed in the gastrointestinal tract of humans and animals. Bifidobacteria are among the first colonizers of the human intestinal tract after birth (14, 45) and in breast-fed infants are one of the predominant groups of the colonic microbiota (37). Although their numbers decrease after weaning, they still represent an important group of intestinal bacteria. While 16S rRNA gene-based studies suggest that bifidobacteria represent only a minor population of the colonic and fecal microbiota (16, 21), a recent publication has revealed that the universal primers used in these studies have a mismatch of bases to the bifidobacterial 16S rRNA genes (47). This probably leads to an underrepresentation of bifidobacteria in the 16S libraries due to less efficient amplification of their 16S genes. When looking at metagenomic libraries that take into account the entirety of genetic information rather than just 16S rRNA gene sequences, *Actinobacteria*, which in the human intestinal microbiota are represented almost exclusively by bifidobacteria, become again the third most abundant group of colonic microorganisms, surpassed only by the *Bacteroidetes* and *Firmicutes* (37, 67), confirming earlier culture-based studies (84).

Diverse beneficial effects regarding the health status of the human host have been reported for bifidobacteria. These include prevention of diarrhea, establishment of a healthy microbiota, alleviation of constipation, lactose tolerance, cholesterol reduction, treatment of inflammatory disorders of the gastrointestinal tract (GIT), immunostimulation, and cancer prevention (reviewed in references 38 and 39). More recently, the protective effect of bifidobacteria against infections with Gram-negative pathogens has received considerable attention (17, 20).

The nonpathogenic nature of bifidobacteria and their status as "generally recognized as safe" (GRAS) organisms have made them interesting candidates for the delivery and production of therapeutic genes and proteins for cancer therapy. The idea of using bacteria as vectors for cancer therapy dates back to 1955, when Malmgren and Flanigan could show that vegetative *Clostridium tetani* cells are found in tumor tissue after intravenous administration of spores (46). Over the years, different bacteria have been tested as tumor-targeting vectors, including members of the genera *Escherichia*, *Salmonella*, *Clostridium*, *Caulobacter*, *Listeria*, *Proteus*, *Streptococcus*, and *Bifidobacterium* (4, 18). A number of studies have employed bifidobacteria as vectors in tumor targeting for delivery of therapeutic genes, tumor drugs, or prodrug-converting enzymes (reviewed in reference 83). Bifidobacteria have been shown to selectively colonize solid tumors in animal models (9, 28). Recently, Cronin and colleagues could show for the first time that oral administration of *B. breve* UCC2003 to mice resulted in bifidobacterial translocation from the GIT and subsequent homing to and replication specifically in tumors at levels similar to those found with intravenous administration (9, 11). This suggests that bifidobacteria translocate across the intestinal epithelium and spread systemically without causing severe side effects.

Another promising biomedical application of bifidobacteria is their use as live vaccines. A few studies have been recently performed using recombinant bifidobacterial expression antigens of pathogenic bacteria as live vaccines, and initial results in animals have indicated the potential of this new vaccination strategy (43, 80, 87).

Published ahead of print 11 May 2012

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doi:10.1128/AEM.00551-12

The use of bifidobacteria as tumor-targeting vectors or as live vaccines offers several advantages over the use of other bacteria. First of all, bifidobacteria colonize the intestinal tract of human breast-fed newborns at high numbers (14, 37, 45) without any adverse effects. Additionally, they have a long history of documented safe use in infant formulas and other probiotic preparations. Also, as Gram-positive organisms they do not express pyrogenic lipopolysaccharides.

Due to these properties, bifidobacteria have attracted increasing commercial interest and are frequently used in probiotic preparations. However, bifidobacteria are notoriously resistant to genetic modification and only very few reports on directed mutagenesis are available. Thus, the molecular mechanisms responsible for their beneficial effects on human health are largely unknown. Moreover, only a few well-characterized molecular tools for the generation of recombinant strains are available, hampering the development of tailored vectors for the delivery of therapeutic genes, drugs, or enzymes. In this review, we provide an overview of the currently available transformation protocols, plasmids, and determinants of expression as well as successfully expressed genes and their applications.

TRANSFORMATION PROTOCOLS

A number of Gram-positive organisms, e.g., *Bacillus subtilis* and *Streptococcus pneumoniae*, possess systems for the uptake of foreign DNA, making them naturally competent for transformation (8). Despite the fact that the sequenced bifidobacterial genomes encode a few genes with limited homology to the genes of the competence systems of other organisms, natural competence has not been observed in bifidobacteria to date. Additionally, Gram-positive microorganisms are enveloped by a thick and complex cell wall of peptidoglycan, lipoteichoic and wall teichoic acids and proteins, and additional surface layers, such as capsules or loosely attached exopolysaccharides. Due to this physical barrier and the presence of restriction/modification systems, genetic transformation of bifidobacteria is more challenging than for bacteria that possess competence systems.

To date, several protocols for transformation of bifidobacteria are available. All of these protocols are based on electroporation for DNA transfer; they usually work only for a limited number of strains or species, and transformation efficiencies are generally very low. The first protocol published for electrotransformation of a *B. longum* strain with pRM2, an *Escherichia coli*/*Bifidobacterium* shuttle vector, yielded less than 4×10^2 CFU per μg of plasmid DNA (49). In another study, the protocol for electroporation was optimized for a *B. animalis* strain by adding sucrose to the MRS medium and the washing buffer used for preparation of electrocompetent cells (3). The use of a transformation buffer containing citrate, an optimized voltage, and an extended incubation at 4°C prior to electroporation resulted in further improvements (3). This protocol was subsequently used to successfully electroporate other bifidobacteria with transformation efficiencies ranging from 2×10^2 to 7×10^4 CFU/μg plasmid DNA, depending on the strain and species. Similarly low efficiencies were obtained in another study in which several carbon sources used as supplements to the growth medium were tested (73). Other authors have more or less successfully used these protocols or slight variations thereof.

Surprisingly, there have been no reports describing a systematic testing of further parameters since the initial protocols have been published. The use of lysozyme and/or sublethal concentrations of antibiotics that inhibit cell wall synthesis to prepare competent bacteria has been reported for other Gram-positive organisms (50, 66). Pretreatment of *Lactococcus lactis* with lithium acetate and dithiothreitol improved transformation efficiencies by 4 orders of magnitude from approximately 1×10^5 to 2×10^9 (58). However, cell wall-altering treatments have not been tested so far for bifidobacteria, leaving enough room to further improve transformation efficiencies.

Recently, a number of research groups have used another approach to increase transformation efficiencies. In most bacteria, uptake of foreign DNA is limited by restriction modification systems that recognize and degrade foreign DNA, which is not methylated with the recipient-specific pattern. This is illustrated by the fact that electroporation efficiencies are dramatically improved when plasmid DNA is isolated and subsequently reintroduced into the same host (55, 89). By expressing DNA methylases of the restriction/modification systems of the target organisms in methylase-deficient *E. coli* host strains, shuttle vectors can be methylated in the correct pattern and are thus prevented from degradation. Using this approach, transformation efficiencies of *B. breve* and *B. adolescentis* strains were significantly improved (55, 89). Similarly, *in vitro* methylation of plasmids resulted in increased transformation efficiency of *B. longum* (35).

Nevertheless, efficient transformation protocols remain a major obstacle in bifidobacterial research. Since high transformation efficiencies are a prerequisite for the use of suicide vectors, it is not surprising that so far, repeated directed insertional mutagenesis has been reported only for a single strain of *B. breve* with transformation efficiencies of approximately 1×10^7 CFU/μg plasmid DNA (54, 56, 64, 65, 75). More recently, targeted gene disruption of a fructose transporter was reported for a *B. longum* strain by electroporation with a derivative of pBluescript (20). This widely used cloning vector harbors a replicon of an *E. coli* plasmid, which is not functional in bifidobacteria. The protocol used to prepare electrocompetent cells was described earlier (53); however, no information on transformation efficiencies is available. Nevertheless, the authors report on PCR confirmation of the mutant, and thus the results imply that transformation efficiencies were high enough to allow homologous recombination events to occur. Whether the protocol used consistently yields transformation efficiencies high enough to allow for chromosomal integration of suicide vectors needs to be confirmed in further studies.

PLASMIDS

The isolation and characterization of plasmids from bifidobacteria not only are important to understand the genetics of this genus but also are necessary for the development of efficient genetic tools and gene transfer systems. These tools are a prerequisite for the analysis of the beneficial effects of bifidobacteria by mutagenesis or overexpression of specific genes. Naturally occurring plasmids are not commonly found in the genus *Bifidobacterium*, with only 20% of the isolates harboring plasmids (39). In early studies, the presence of plasmids in the genus *Bifidobacterium* has been demonstrated in only a few species (30, 77). Since then, significant efforts have been made to isolate and characterize plasmids from bifidobacteria. To date, plasmids have been isolated from 7 of the 32 species and 34 plasmids have been fully sequenced (Table 1). More than half of the sequenced plasmids were isolated from *B. longum* subsp. *longum*, with a GC content ranging from 59.0% to

TABLE 1 Completely sequenced bifidobacteria, plasmids, and accession numbers

Species	Plasmid	Accession no.[a]
B. longum	pNAC2	AY112723.1
	pTB6	NC_006843.1
	pB44	AY066026.1
	pKJ36	AF139129.1
	pMG1	NC_006997.1
	pBLO1	AF540971.1
	p6043B	DQ458911
	pNAC1	AY112724.1
	pNAL8L	AM183145.1
	pKJ50	U76614.1
	pNAL8M	AM183144.1
	pBIFA24	NC_010164.1
	p6043A	DQ458910
	pNAC3	AY112722.1
	pDOJH10L	AF538868.1
	pDOJH10S	AF538869.1
	pMB1	X84655
	pSP02	GU256055.1
	pFI2576	NC_011139.1
	BLNIAS_P1	CP002795.1
	BLNIAS_P2	CP002796.1
	p157F-NC1	AP010891.1
	p157F-NC2	AP010892.1
	pBK283	AB495342.1
B. breve	pCIBb1	AF085719.1
	pNBb1	E17316
	pB21a	NC_010930.1
B. pseudolongum subsp. *globosum*	pASV479	NC_010877.1
B. bifidum	pB80	NC_011332.1
	pBIF10	DQ093580
B. asteroides	pCIBAO89	NC_010908.1
	pAP1	Y11549
B. catenulatum	pBC1	NC_007068.1
B. pseudocatenulatum	p4M	AF359574.1

[a] Accession numbers are for the NCBI nucleotide database.

66.2%. Other sources include *B. breve*, *B. pseudolongum* subsp. *globosum*, *B. bifidum*, *B. asteroides*, *B. catenulatum*, *B. indicum*, and *B. pseudocatenulatum* (Table 1).

Although a number of plasmids have been characterized in bifidobacteria, their significance still remains largely unknown, as no obvious phenotypic properties have been associated with their presence except for a putative *B. bifidum* plasmid, which is suspected to harbor genes responsible for the production of a bacteriocin (90). Native plasmids of bifidobacteria and their replicons are mainly used for the construction of *E. coli*/*Bifidobacterium* shuttle vectors aiming to overcome the lack of molecular tools for bifidobacteria (1, 10, 36, 40, 48, 49, 71, 72, 76, 79).

Bacterial plasmids replicate by either the rolling circle or the theta mechanism. Both mechanisms were proposed for different plasmids isolated from bifidobacteria. For example, pCIBb1, a plasmid isolated from a *B. breve* strain (57), and pKJ50, isolated from a *B. longum* strain (60), are thought to replicate by the rolling circle mechanism. For the *B. longum* plasmid pDOJH10S and pCIBA089 isolated from a *B. asteroides* strain, the theta mechanism of replication has been suggested (10, 40). Predicted origins of transfer and mobilization proteins are frequently found in plasmids isolated from bifidobacteria, suggesting that they are mobilizable. This raises the question of whether genetic modification by means of conjugation is possible in bifidobacteria.

Of the plasmids isolated from bifidobacteria, the replicons most frequently used in *E. coli*/*Bifidobacterium* shuttle vectors are those of pTB6, pMB1, pMG1, and pBC1 (Table 2). All four are small cryptic plasmids, with the former three isolated from *B. longum* strains and the latter from a *B. catenulatum*. pTB6 harbors four open reading frames, including a gene encoding a RepB protein, suggesting a replication by the rolling circle mechanism (82). This replicon has been successfully used in *B. longum*, *B. breve*, and *B. animalis* for expression of *E. coli* cytosine deaminase or the *Salmonella* FliC protein (26, 27, 53, 80, 87). The replicon of plasmid pMB1 was shown to replicate stably in *B. longum*, *B. animalis*, and *B. adolescentis* by the theta mechanism and was used to express endostatin, tumor necrosis factor (TNF)-related apoptosis-inducing ligand, and human interleukin 10 (19, 28, 41, 68, 71, 86). The replicon of the theta-replicating plasmid pMG1 has been used exclusively in its native host strain *B. longum* MG1 for expression of the rice glutamate decarboxylase, the bacteriocin pediocin, and the cholesterol oxidase of *Streptomyces coelicolor* (52, 59, 61, 62). The theta-type replicon of pBC1 has been shown to replicate stably in *B. pseudocatenulatum*, *B. breve*, and *B. longum* subsp. *infantis* and was used for bioluminescent imaging of *B. breve* and expression of two different antigens of enteropathogenic *E. coli* in a *B. longum* subsp. *infantis* strain (2, 9, 11, 12, 43).

Most bifidobacterial isolates are intrinsically resistant to a range of antibiotics, including vancomycin, gentamicin, kanamycin, streptomycin, nalidixic acid, polymyxin B, and others (5). The most widely used selection markers in bifidobacteria are genes conferring resistance to spectinomycin, erythromycin, chloramphenicol, or ampicillin (Table 2). However, it has to be mentioned that sensitivity to erythromycin, ampicillin, and chloramphenicol has been shown to vary between different strains and species of bifidobacteria (15, 32).

DETERMINANTS OF EXPRESSION

Several studies have reported on successful heterologous gene expression in bifidobacteria. The majority of studies involving recombinant bifidobacteria target cancer treatment or live vaccination by expression of prodrug-converting enzymes, therapeutic proteins, or antigen (Table 2). Depending on the application, more or less fine-tuned expression of the protein of choice is required. Moreover, efficient expression and correct localization of the recombinant gene product depend on a number of factors, including plasmid copy number, promoter strength, regulatory elements, terminators, and localization signals, such as secretion signals, cell wall, and membrane anchor sequence.

CONSTITUTIVE PROMOTERS

In microorganisms, transcription, i.e., mRNA synthesis, is driven by promoter sequences and stops at termination signals. Promoters play a leading role in regulating gene transcription. A number of promoters are currently used for expression of recombinant protein in bifidobacteria. In an early study, a histone-like protein was isolated in large amounts from a heat-treated *B. longum* ex-

TABLE 2 Plasmids used for recombinant protein expression in bifidobacteria

Plasmid	Replicon	Promoter	Selection marker[a]	Protein expressed	Host species	Localization signal	Reference
pBLES100-S-eCD	pTB6	P_{hup}	Spc	*E. coli* cytosine deaminase	*B. longum*		43
pAV001-HU-eCD	pTB6	P_{hup}	Spc	*E. coli* cytosine deaminase	*B. breve*, *B. longum*		21
pAV001-HU-eCD-M968	pTB6	P_{hup}	Spc	*E. coli* cytosine deaminase	*B. longum*		20
pBLES-FliC	pTB6	P_{hup}	Spc	*S. typhimurium* FliC	*B. animalis*		68
pJW245	pTB6	P_{hup}	Spc	*S. typhimurium* FliC	*B. longum*	GltA-FliC fusion for surface display	75
pBV220/endostatin	pMB1	$\lambda P_R P_L$	Amp	Human endostatin	*B. adolescentis*, *B. longum*		15, 35
pBV22210	pMB1	$\lambda P_R P_L$	Cm	Human endostatin	*B. longum*		74
pBV22210-TRAIL	pMB1	$\lambda P_R P_L$	Cm	Human TNF-related apoptosis-inducing ligand	*B. longum*		22
pLR2	pMB1	P_{hup}	Cm	Synthetic human interleukin 10	*B. longum*	β-Galactosidase signal peptide	56
pBIFRIBO-gusA	pASV479	$P_{16SrRNA}$	Cm	β-Glucuronidase	*B. breve*		64
pESH86	pB80	P_{hup}	Erm	Human fibroblast growth factor	*B. breve*	Sec2 signal peptide	66
pESH46	pB44	P_{hup}					
pESH47	pB44	P_{gap}					
pGUSA	pNCC293	P_{gap}	Spc	*E. coli* β-glucuronidase	*B. longum*		30
pGUSB	pNCC293	P_{BL1613}	Spc				
pGUSC	pNCC293	P_{aga}	Spc				
pPSAB1	pMG1	P_{amy}	Cm	*Pediococcus* spp. pediocin PA-1	*B. longum*	α-Amylase signal peptide	42
pBES16PR-CHOL	pMG1	$P_{16SrRNA}$	Cm	*S. coelicolor* cholesterol oxidase	*B. longum*		50
pESH92	pB80	P_{hup}	Erm	Human interleukin 10	*B. breve*	Sec2 signal peptide	27
pESH93	pB80	P_{gap}	Erm	Human interleukin 10	*B. breve*	Sec2 signal peptide	
pESH99	pB80	P_{gap}	Erm	Human interleukin 10	*B. breve*	Sec2 signal peptide	
pESH100	pB80	P_{gap}	Erm	Human interleukin 10	*B. breve*	AmyB signal sequence	
pESH101	pB80	P_{gap}	Erm	Human interleukin 10	*B. breve*	ApuB signal sequence	
pESH102	pB80	P_{gap}	Erm	Human interleukin 10	*B. breve*	Sec2 signal peptide	
pESH103	pB80	P_{gap}	Erm	Human interleukin 10	*B. breve*	Sec2 signal peptide	
pLuxMC2	pBC1	P_{rep}	Cm	*Photorhabdus luminescens* luciferase	*B. breve*		10
pLuxMC3	pBC1	P_{help}	Cm	*P. luminescens* luciferase	*B. breve*		
pBEX–CfaB	pBC1	P_{amy}	Amp	Enterotoxigenic *E. coli* CfaB	*B. infantis*		37
pBEX–LTB	pBC1	P_{amy}	Amp	Enterotoxigenic *E. coli* LTB	*B. infantis*		
pMDYP469AbfB	pNCC293	P_{betA}	Cm	*B. longum* arabinofuranosidase	*B. longum*, *B. breve*, *B. adolescentis*, *B. pseudocatenulatum*	Bile inducible	62
pGBL8b	pNAL8L	T5	Cm	Firefly luciferase	*B. longum*		19
pBBADs-OXM	?	P_{araC}	Amp	Human oxyntomodulin	*B. longum*	XynF signal peptide	36
pBBADs-IL-10	?	P_{araC}	Amp	Human interleukin 10	*B. longum*	XynF signal peptide	76
pBBADs-IL-12	?	P_{araC}	Amp	Human interleukin 12	*B. longum*	XynF signal peptide	79

[a] Spc, spectinomycin; Amp, ampicillin; Cm, chloramphenicol; Erm, erythromycin.

tract (22) and, thus, its promoter P_{hup} was expected to show high transcriptional activity. While no data are available on the actual transcriptional activity of P_{hup} compared to those of promoters of housekeeping genes, this promoter was used in a number of studies for the expression of bacterial cytosine deaminase (26, 27, 53) and flagellin (87) or human interleukin 10 (33, 68) and fibroblast growth factor (78) in different *B. longum*, *B. breve*, and *B. animalis* strains.

A convenient tool for the identification of promoter sequences and the determination of their transcriptional activity in bifidobacteria is the reporter plasmid pMDY23, which uses the *E. coli gusA* gene for promoter probing (36). Using pMDY23, the transcriptional activity of three promoters of a *B. longum* strain was tested. The corresponding genes of these promoters showed high, low, or inducible expression in microarray experiments, which was confirmed using pMDY23 constructs. These results corroborate that *gap*, i.e., the gene encoding glyceraldehyde-3-phosphate dehydrogenase, is highly expressed in bifidobacteria. Moreover, the data suggest that the promoter driving expression of the *B. longum* α-galactosidase is inducible by raffinose and therefore could be used for inducible expression of recombinant protein in at least *B. longum* strains. The *gap* promoter (P_{gap}) was subsequently used in two studies for the expression of human interleukin 10 and fibroblast growth factor in *B. breve* (33, 78). Of note, the transcriptional activity of P_{gap} was enhanced significantly by introduction of the signal sequence of the secreted α-amylase ApuB of *B. breve*, resulting in increased protein production possibly by enhanced promoter activity or mRNA stability (33).

Since P_{hup} and P_{gap} do not harbor any regulatory elements and show transcriptional activity under standard laboratory conditions, they can be considered constitutive. Other bifidobacterial promoters used for constitutive recombinant protein expression in bifidobacteria are P_{amy}, the promoter of the α-amylase gene of a *B. adolescentis* strain (43, 52), and the promoter of the 16S rRNA gene (61, 76). Promoter sequences of other organisms that have been used successfully for expression in bifidobacteria are P_{help} (12), a synthetic Gram-positive consensus promoter designed for constitutive high-level expression in *Listeria monocytogenes* (70) and Gram-negative bacteria (69), and the lambda phage promoter $P_R P_L$ (19, 28, 41, 86).

INDUCIBLE PROMOTERS

In order to establish systems for controlled gene expression, promoters are needed for which transcriptional activity can be induced or repressed. However, only limited information is available with respect to regulated gene expression in bifidobacteria. Using the promoter probe vector pMDY23, the promoter driving expression of the α-galactosidase gene of *B. longum* NCC2705 was shown to be induced in the presence of raffinose and repressed by glucose (36). However, this promoter has not been used to express genes other than the reporter. Using transcriptomics, a large number of genes were identified in the stress response of a *B. breve*

strain to heat, ethanol, or osmotic shock (92). Similarly, a bile-inducible efflux transporter was identified in *B. longum* NCC2705 (24) and the promoter region was further characterized and shown to function as a bile-responsive element in strains of the species *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. breve*, *B. pseudocatenulatum*, and *B. adolescentis* using the GusA reporter system encoded by pMDY23 (74). Thus, this promoter might be suitable for inducible expression of recombinant proteins in bifidobacteria.

In the context of inducible expression, it has to be mentioned that several studies have successfully used the arabinose-inducible *araC*-P_{BAD} expression system of *E. coli* in *B. longum* (42, 88, 91). However, it has to be noted that not all bifidobacteria are able to ferment arabinose (63) possibly due to the lack of functional arabinose transport systems. Thus, the use of the P_{BAD} system might be limited to species and strains that are able to utilize arabinose. Nevertheless, successful use of the P_{BAD} system in bifidobacteria suggests that at least some *E. coli* promoters are recognized by the transcription machinery of bifidobacteria. This is supported by the use of the T5 phage promoter for recombinant protein expression in bifidobacteria (25, 42) and P_{hup} for expression of a codon-optimized human interleukin 10 gene in both *E. coli* and *B. longum* (68).

TERMINATORS

Terminators are commonly used in expression vectors for efficient expression, since they ensure the proper termination of mRNA synthesis, thus avoiding additional biosynthetic burden on the expression host. Transcription terminators are signals for the RNA polymerase to cease transcription of a DNA template. In bacteria, two general termination mechanisms are characterized. The rho-independent transcription termination (also called intrinsic termination) involves terminator sequences at the end of the mRNA. Usually, the terminator sequence is a palindromic motif leading to formation of a hairpin (or stem-loop) structure of the transcribed mRNA molecule which is followed by a number of uracil residues. This structure of the mRNA molecule leads to the dissociation of the RNA polymerase from the DNA template, thus terminating transcription. Rho-dependent termination is dependent on the rho factor protein, which blocks RNA synthesis at specific sites. Most Rho-dependent terminators have been found in Gram-negative organisms, but there are a few examples in Gram-positive bacteria (7). The only two terminator sequences so far described for bifidobacteria are rho independent (29, 34). There is only very limited information on the use of terminator sequences with respect to recombinant protein expression in bifidobacteria. Shkoporov et al. used the terminator sequence of the *hup* gene for expression of human fibroblast growth factor but did not report on its efficacy (78).

LOCALIZATION SIGNALS

To properly perform the intended function and to achieve a maximum of efficacy, it is essential to ensure that recombinant proteins are expressed at the correct location of a bacterial cell. This is achieved either by the addition of particular signal sequences that target the protein to the desired cellular compartment or by secretion to the external environment. In principle, a protein can be localized to four different compartments: cytoplasm, membrane, bacterial surface, or surrounding environment. In the context of therapeutic proteins and live vaccines, only signals leading to secretion to the external environment or surface display by covalent or noncovalent linkage to membrane or cell wall components are of relevance.

Bifidobacteria have been used for expression of recombinant proteins, which usually would require secretion or surface display in order to interact with host cells and exert an effect. However, in a number of studies *E. coli* cytosine deaminase (26, 27, 53), *Salmonella enterica* FliC (80), proteins of enterotoxigenic *E. coli* (43), and endostatin (19, 41, 86) were expressed without providing a signal for secretion or surface display. Nevertheless, the recombinant bifidobacteria have yielded the desired effects at least partially. In these cases it has to be assumed that effects are due to release of recombinant protein upon bacterial cell lysis or phagocytosis by host cells.

MacConaill and colleagues have used a genomic library of a *B. breve* strain to screen for secretion signals using an export-specific nuclease reporter approach and have identified several putative signal peptides (44). While the identified bifidobacterial signal peptides were longer than those of other Gram-positive organisms, they contained motifs with high similarity to the A-X-A consensus cleavage site (44). Moreover, analysis of the genomes of the *B. breve* strain used in this study and two *B. longum* genomes revealed genes for all components of the Sec pathway commonly found in Gram-positive bacteria (44). In contrast, no components of a Tat protein secretion machinery were found (44). In conclusion, the secretory machinery of at least the three *Bifidobacterium* strains examined appears to be similar to that of other Gram-positive bacteria.

Bifidobacterial secretion signals have been used by a number of studies to ensure export of recombinant protein. For example, the signal peptide of the α-amylase of *B. adolescentis* INT-57 was used to express the *Pediococcus* spp. class II bacteriocin pediocin PA-1 in *B. longum* MG1, and the recombinant protein was found in the supernatants of the recombinant strain and was active against *Lactobacillus plantarum* and *Listeria monocytogenes* (52). The signal peptide of the β-galactosidase was successfully used for secretion of human interleukin 10 by *B. longum* (68). A series of expression vectors carrying the *B. breve* Sec2 signal peptide was constructed. Functionality of the constructs was proven by expression of human fibroblast growth factor 2 and interleukin 10 and detection of recombinant proteins in the supernatants (33, 78). Khokhlova et al. also used the *B. breve* AmyB and *B. adolescentis* ApuB signal sequences in combination with different promoters for the expression of human interleukin 10 and found a surprising increase in expression on both mRNA and secreted protein levels with the AmyB signal compared to the Sec2 signal peptide (33). The *B. longum* XynF signal peptide was successfully used to express and export recombinant human oxyntomodulin and interleukins 10 and 12 (42, 88, 91).

Surface display of recombinant protein has been used far less. The only report so far describing deliberate use of an anchor sequence for surface display is the study by Yamamoto et al. The authors created a fusion of *gltA*, the gene encoding the substrate-binding protein of an ABC transporter of *B. longum* JCM1217, to *fliC*, the gene encoding the *S. enterica* flagellin. The fusion protein was successfully expressed, as shown by Western blotting and fluorescence microscopy. However, correct location of the fusion protein on the surface was not verified by testing different fractions, e.g., cytoplasm membrane and cell wall, of the recombinant strain (87).

CONCLUSIONS

While bifidobacteria have attracted considerable commercial interest due to their beneficial probiotic properties, the molecular tools to study their physiology and genetic traits underlying their beneficial effects are largely missing. The gold standard in microbiology to demonstrate the function of a gene and its product is the generation of knockout mutants. Transformation efficiencies of bifidobacteria are generally below the minimum required for use of suicide vectors to generated mutants by targeted genetic recombination. The notoriously low transformation efficiencies of other bifidobacteria might be overcome by systematically testing and applying cell wall-modifying treatments that have yielded improved transformability in other Gram-positive bacteria, including lysozyme, mutanolysin, antibiotics targeting cell wall synthesis, lithium acetate, or dithiothreitol.

The only exception so far for which transformation efficiencies have been repeatedly sufficient to generate insertional mutations is *B. breve* UCC2003. This is achieved by methylation of the integration vectors in a recombinant *E. coli* host strain expressing the DNA methyltransferases of the target organism *B. breve* UCC2003. Target-specific methylation of plasmids has been successfully applied to a number of organisms (6, 23, 51) and thus represents an alternative to improve transformation efficiencies of bifidobacteria.

Genome sequencing and analysis have revealed a number of insertion elements and prophages. However, to the best of our knowledge we are not aware of transposon systems for random mutagenesis of bifidobacteria. Thus, there is significant need for efficient transformation systems and tools for targeted and random mutagenesis for bifidobacteria.

While it is reasonable to use standard antibiotic selection markers for mechanistic studies on the probiotic properties of bifidobacteria, their application for expression of recombinant protein is difficult for several reasons. The use of antibiotics is not applicable in industrial-scale production of recombinant strains due to their high costs and difficulties in complete removal during downstream processing. Also, it is problematic to use recombinant bifidobacteria harboring antibiotic resistance markers as vaccine strains or delivery vectors for therapeutic proteins due to the possibility of horizontal transfer of resistance genes to commensal and pathogenic bacteria in various habitats of the host. Moreover, the introduction of foreign antibiotic genes into probiotics is in conflict with the food-grade standard. The development and use of nonantibiotic selection markers such as levansucrase (81) or glucosamine synthase (85) in bifidobacteria might overcome these limitations. Additionally, nonantibiotic markers might also offer the possibility of circumventing the poor transformation efficiencies of bifidobacteria by forcing integration of a nonsuicide vector at the desired locus. Moreover, once integrated into the chromosome, the same gene can be used as a counterselection marker to force loss of the plasmid. Thus, another advantage of these nonantibiotic selection markers is the sequential mutagenesis of several genes using the same marker.

Regarding recombinant protein expression, it has to be mentioned that high-level expression as shown for other organisms, e.g., *E. coli*, has not been reported so far for bifidobacteria. To date no study has shown protein expression by SDS-PAGE. Only very few studies show protein data at all, and in most cases, expressed protein can be detected only by Western blotting using either antibodies specific for the expressed protein or targeting an artificially fused polyhistidine tag. This indicates that levels of expressed recombinant protein are generally very low, which in turn leaves plenty of room for significant improvements by novel expression systems. The most obvious way to improve levels of expressed proteins is to choose or design the right promoter. Further studies of the transcriptional activity of promoters relative to other promoters, for example by transcriptional analysis on a genomewide level, might help to identify highly active constitutive or tightly regulated promoters leading to the development of better genetic tools. One such example is the recently published study by Cronin et al., who used microarray analysis to identify iron-regulated genes and used the results to create an iron-inducible expression system (13). However, while the recently published bile- and iron-inducible promoters will prove valuable tools to study the role of individual proteins on bifidobacterial physiology *in vitro* and *in vivo*, they are probably of limited use for the generation of recombinant strains expressing tumor therapeutics or vaccine antigens.

Large-scale analysis of promoter sequences identified in the sequenced genomes could be used to formulate bifidobacterial consensus promoter sequences and, together with transcriptomic analysis, could be used to generate synthetic promoters with various levels of transcriptional activities, as shown for the *Lactococcus lactis* consensus promoter (31).

Another way of optimizing expression levels is to use replicons with the desired copy number. Copy numbers of only a few replicons have been characterized (1, 2, 10, 36) and were shown to depend on the host strain (2). The determination of plasmid copy numbers is particularly important to distinguish the effect of the promoter on expression levels from that of the gene dosage, which is linked to plasmid copy number.

While some progress has been made in recent years with respect to the development of expression vectors, there is still a need for generation of efficient genetic tools for bifidobacteria. These tools are required for the functional analysis of the mechanisms employed by bifidobacteria to colonize the host and exert health-promoting effects and the generation of recombinant strains expressing therapeutic proteins, vaccine antigens, or proteins improving the probiotic properties.

ACKNOWLEDGMENTS

Z.S., D.Z., J.Y., and C.U.R. are supported by the German Academic Exchange Service/Federal Ministry of Education and Research (grant D/09/04778).

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